

DTIC FILE COPY

Best Available Copy

(2)

AD-A220 814

AD _____

THERAPEUTIC APPROACHES TO THE
TREATMENT OF BOTULISM

Annual Report

October 1, 1989

Lance L. Simpson

DTIC
ELECTE
APR 23 1990
S D D

Supported by:

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-85-C-5285

Jefferson Medical College
Thomas Jefferson University
1025 Walnut Street
Philadelphia, Pennsylvania 19107

Approved for public release; distribution unlimited

The findings in this report are not to be construed as
an official Department of the Army position unless so
designated by other authorized documents.

20030206267

90 04 17 025

Best Available Copy

REPORT DOCUMENTATION PAGE

Form Approved
OMB No 0704-0188
Exp Date Jun 30, 1986

1a REPORT SECURITY CLASSIFICATION Unclassified		1b RESTRICTIVE MARKINGS	
2a SECURITY CLASSIFICATION AUTHORITY		3 DISTRIBUTION/AVAILABILITY OF REPORT Approved for public release; distribution unlimited	
2b DECLASSIFICATION/DOWNGRADING SCHEDULE		5 MONITORING ORGANIZATION REPORT NUMBER(S)	
4 PERFORMING ORGANIZATION REPORT NUMBER(S)		7a NAME OF MONITORING ORGANIZATION	
5a NAME OF PERFORMING ORGANIZATION Jefferson Medical College	6b OFFICE SYMBOL (if applicable)	7b ADDRESS (City, State, and ZIP Code)	
5c ADDRESS (City, State, and ZIP Code) 1025 Walnut Street Philadelphia, PA 19107		7c ADDRESS (City, State, and ZIP Code)	
8a NAME OF FUNDING/SPONSORING ORGANIZATION US Army Medical Research & Development Command	8b OFFICE SYMBOL (if applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER DAMD-85-C-5285	
8c ADDRESS (City, State, and ZIP Code) Fort Detrick Frederick, MD 21701-5012		10 SOURCE OF FUNDING NUMBERS	
		PROGRAM ELEMENT NO 62787A	PROJECT NO 3MI
		TASK NO AA	WORK UNIT ACCESSION NO 393
11. TITLE (Include Security Classification) (U) Therapeutic Approaches to the Treatment of Botulism.			
12. PERSONAL AUTHOR(S) Lance L. Simpson			
13a TYPE OF REPORT Annual	13b TIME COVERED FROM 9/1/88 TO 7/5/89	14 DATE OF REPORT (Year, Month, Day) 1989 October 1	15 PAGE COUNT 24
16 SUPPLEMENTARY NOTATION			
17 COSATI CODES		18 SUBJECT TERMS (Continue on reverse if necessary and identify by block number)	
FIELD	GROUP	SUB-GROUP	
06	16		
06	15		
		Botulinum neurotoxin; neuromuscular blockade; experimental therapeutics, RAI (A-T)	
19 ABSTRACT (Continue on reverse if necessary and identify by block number)			
<p>Work during the past year has focused on three problems:</p> <p>i.) aminopyridines and their analogues, ii.) dendrotoxin, and iii.) rubidium flux. The work on aminopyridines confirms that they are very narrow in their utility as anti-botulism agents. The studies on dendrotoxin resulted in the provocative finding that the agent did not antagonize any clostridial neurotoxin, nor could it reverse the effects of low calcium or high magnesium. The final aspect of the research was to establish a protocol for studying rubidium flux. This may represent a rational approach for finding clostridial toxin antagonists.</p>			
20 DISTRIBUTION/AVAILABILITY OF ABSTRACT <input checked="" type="checkbox"/> UNCLASSIFIED/UNLIMITED <input checked="" type="checkbox"/> SAME AS RPT <input type="checkbox"/> DTIC USERS		21 ABSTRACT SECURITY CLASSIFICATION Unclassified	
22a NAME OF RESPONSIBLE INDIVIDUAL Mary Frances Bostian		22b TELEPHONE (Include Area Code) 301-663-7325	22c OFFICE SYMBOL SGRD-RMT-S

FOREWARD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHEW Publication No. (NIH) 86-23, Revised 1985).

Citations of commercial organizations and trade names in this report do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

Accession For		
NTIS	CRA&I	<input checked="" type="checkbox"/>
DTIC	TAB	<input type="checkbox"/>
Unannounced		<input type="checkbox"/>
Justification		
By		
Distribution /		
Availability Codes		
Dist	Avail and/or Special	
A-1		

TABLE OF CONTENTS

FOREWORD	1
TABLE OF CONTENTS	2
STATEMENT OF PROBLEM	3
STUDIES ON AMINOPYRIDINES.....	3
STUDIES ON DENDROTOXIN.....	10
STUDIES ON RUBIDIUM FLUX.....	14
DISTRIBUTION LIST.....	24

1. Statement of Problem

Pharmacological methods are being sought to prevent or reverse the effects of botulinum neurotoxin. During the past year, emphasis has been placed on drugs that interact with potassium channels. *can 42 pg 001473*

2. STUDIES ON AMINOPYRIDINES

A. Background

Recent work by the Principal Investigator and by others has drawn attention to a number of anomalies. First, 4-AP and its analogs are potassium channel blockers, and this secondarily promotes calcium influx and acetylcholine efflux. An action like this would be predicted to antagonize botulinum neurotoxin. However, 4-AP and its analogs are strong antagonists of only serotype A.

A second anomaly pertains to dendrotoxin. This substance is also a potassium channel blocker, and thus it too would be expected to antagonize toxins that block exocytosis. Indeed, when tested against beta-bungarotoxin it did delay onset of paralysis (see below). But when tested against other phospholipase neurotoxins (e.g., crotoxin), it did not afford protection.

Finally, there is a "crossed anomaly". 4-AP can protect against at least one of the serotypes of botulinum neurotoxin, but it does not protect against any of the phospholipase neurotoxins. Conversely, dendrotoxin can protect against beta-bungarotoxin, but it does not protect against any of the clostridial toxins (again, this report).

The present study provides data from a large scale screening process in which various putative antagonists were tested against various neuromuscular blocking agents. These data are a prelude to trying to unravel the basis for the numerous apparent anomalies.

B. Methods

The techniques used during the past year have been described in previous reports.

C. Results

The interaction between 4-AP (and 3,4-DAP) and clostridial neurotoxins has been adequately described in the literature. The present report will focus on three types of interaction: i.) 4-AP and phospholipase A2 neurotoxins, ii.) dendrotoxin and clostridial neurotoxins, and iii.) potassium channel blockers and magnesium.

1. 4-AP and PLA2 Neurotoxins.

Three snake neurotoxins were tested: beta-bungarotoxin (obtained commercially), crotoxin (isolated in the Principal Investigator's lab), and notexin (obtained from a collaborator). Each was titrated on the mouse phrenic nerve-hemidiaphragm preparation to produce an eventual paralysis time of 100 to 120 minutes.

Two groups of tissues were then exposed to equiactive concentrations of toxin. A control group was treated only

with toxin; an experimental group was titrated with 4-AP to produce at least a 50% enhancement in muscle twitch (conc. $\sim 10^{-4}$ M). The data (Table 1) show that 4-AF was not an effective antagonist against any of the PLA2 neurotoxins.

2. Dendrotoxin and Clostridial Neurotoxins.

Similarly to the previous series of experiments, the clostridial neurotoxins were added to tissues at concentrations that produced paralysis in 100 to 120 minutes. Types A, B, and E neurotoxin were tested. Type E was activated with trypsin before addition to neuromuscular preparations. As an internal control, experiments were also done with dendrotoxin and beta-bungarotoxin.

As expected, dendrotoxin (Table 2) was an antagonist of beta-bungarotoxin. When tissues ($n=5$) were exposed only to the PLA2 neurotoxin, the eventual paralysis times were 117 ± 14 min. (Table 2). This was in marked contrast to the findings with the clostridial neurotoxins. In the latter case, dendrotoxin never afforded protection.

3. Potassium channel blockers and magnesium.

Magnesium is an effective neuromuscular blocking agent whose mechanism of action is well known: it is a competitive antagonist of calcium. When calcium levels in physiological solution are lowered (1.0 mM), increases in the levels of magnesium (10^{-15} mM) will paralyze transmission.

Individual tissues were paralyzed by lowering calcium and increasing magnesium. Tissues were then treated with 4-AP or with dendrotoxin. The results (Table 3) showed an interesting outcome. 4-AP was able to completely overcome Mg-induced blockade, but dendrotoxin was almost completely ineffective.

Although additional experiments are needed, the results with magnesium suggest that there may be a "mislabeling" in the literature. Although dendrotoxin does block potassium channels and does enhance calcium flux, this is not a major action. It is not, for example, an action that is capable of overcoming magnesium-induced block. Most probably, there is some other action that accounts for the ability of dendrotoxin to antagonize beta-bungarotoxin. One possibility is competition for a common binding site.

The same may be true for 4-AP and its analogues. It purportedly antagonizes type A botulinum toxin by virtue of being a potassium channel blocker. However, this is an hypothesis that has not been proved, and other explanations are possible.

TABLE 1

The Interaction Between 4-AP
and PLA2 Neurotoxins

Toxin	Paralysis Time ¹	
	Control ²	4-AP ²
Beta-Bungarotoxin	109±8	111±12
Crotoxin	101±9	108±7
Notexin	117±13	105±4

¹ Minutes (Mean ± SEM)² Group N=5 or more

TABLE 2
The Interaction Between Dendrotoxin
and Presynaptic Neurotoxins

Toxin	Paralysis Time ¹	
	Control ²	Dendrotoxin ²
Beta-Bungarotoxin	117A13	161A14 ³
Botulinum Toxin-A	121±8	116±3
Botulinum Toxin-B	107±5	116±13
Botulinum Toxin-E	112±9	120±14

¹Minutes

²Group N=5 or more

³Significantly different from control (p<0.01)

TABLE 3
The Interaction Between 4-AP or
Dendrotoxin and Magnesium

Time ¹	Treatment ²		
	None	4-AP	Dendrotoxin
1	0	0	0
2	0	1	0
4	1	7	0
8	0	62	1
16	3	139	5
32	3	151	7

¹ Minutes

² Tissues were paralyzed with magnesium, then treated as indicated. The results are expressed as percent of control twitch before addition of magnesium.

3. STUDIES ON DENDROTOXIN

A. Background

During the past year an effort has been made to clarify the mechanism by which drugs that alter potassium channel conductance can act as botulinum neurotoxin antagonists. This work has two motives. Firstly, there has been an assumption that the various serotypes of botulinum neurotoxin have essentially the same mechanisms of action. However, this assumption has been challenged by a variety of experimental findings, including those on 4-aminopyridine (4-AP) and its analogs. These drugs act on potassium channels to increase conductance, secondarily promoting influx of calcium and efflux of acetylcholine. 4-AP is a very effective antagonist of botulinum neurotoxin type A, But it is only weakly active or inactive against the other serotypes. Therefore, one motive for the work has been to determine why only one of the serotypes is strongly antagonized.

A second motive pertains to therapeutics. If one could determine the relationship between 4-AP and type A toxin, that could serve to point the way toward identifying drugs that would have similar relationships to the other serotypes.

B. Methods

The techniques used during the past year have been described in previous Reports.

C. Results

4-AP is regarded as a broad spectrum inactivator of potassium channels. There are other drugs that act more narrowly. The goal of the work during this quarter was to identify a drug that acted on potassium channels, that promoted calcium influx and acetylcholine efflux, but which did not act as a botulinum neurotoxin type A antagonist. This would allow for a kind of pharmacologic algebra. The channels affected by 4-AP minus the channels affected by the drug that is not an antagonist would include a pool of channels that are of importance.

A drug has been identified that satisfies the criteria above. In the initial round of experiments, the venom of *Dendroaspis augustepsis* was tested for its actions on neuromuscular transmission. In agreement with previously published findings by others, the principal investigator found that the venom has a dose dependent action. At low concentrations ($\sim 1 \mu\text{g/ml}$) the venom facilitated transmission. This was manifested by a slowly increasing elevation in the muscle twitch amplitude of nerve-evoked responses (phrenic nerve-hemidiaphragm preparation). As the concentration was increased, so was the magnitude of the enhanced response and the rate at which the effect occurred. At its peak, the muscle response was enhanced about two-fold. With further increases in venom, there was still an enhanced response, but it was not sustained. Instead, the

response waned and eventually the neuromuscular preparation failed.

The venom is known to contain a number of neurotoxins that are referred to generically as dendrotoxins. These toxins are the presumed agents mediating the facilitatory actions of the whole venom. Through the assistance of a collaborator (Dr. R. Sorensen), one of dendrotoxins (I) was isolated and purified to homogeneity. This substance was tested on the isolated neuromuscular junction, and it produced the same spectrum of results as the crude venom.

Dendrotoxin as well as the whole venom were tested for their abilities to antagonize botulinum neurotoxin type A. Individual tissues were titrated with toxin or venom to produce a 50% to 100% increase in response. Botulinum neurotoxin type A (1×10^{-11} M) was then added, and the rate of onset of paralysis was monitored. The results indicated that neither the isolated dendrotoxin nor the whole venom possessed the ability to antagonize botulinum neurotoxin type A. The paralysis times of control tissues and pretreated tissues were essentially identical.

It has been found that even among those drugs that antagonize type A toxin, the effectiveness varies and tends to be highly calcium dependent. Therefore, experiments similar to those above were re-done, but in the presence of elevated calcium (3.6 mM and 7.2mM). The results did not change. Even in the presence of elevated calcium, neither

dendrotoxin nor the whole venom significantly delayed the onset of botulinum neurotoxin type A-induced paralysis.

Dendrotoxin appears to satisfy the criteria discussed earlier. It inactivates potassium channels, it promotes calcium influx and acetylcholine efflux, but it does not antagonize botulinum neurotoxin type A. This means that the channels altered by dendrotoxin must not be the ones through which 4-AP exerts its protective effect. Obviously it would be desirable to identify a drug that acts selectively on the relevant channels.

4. STUDIES ON RIBIDIUM FLUX

A. Background

As explained in previous sections, there has been a convergence of interest directed at potassium channels in nerve cells and especially in nerve endings. There are at least three reasons for this, two of which are important to work conducted under this and an associated contract. To begin with, there are neurotoxin components from various venoms that exert their effects by virtue of interacting with potassium channels. An excellent example of this is dendrotoxin. A second reason, and one of importance to the contract work, is that at least two presynaptically acting neurotoxins are believed to bind wholly or in part to potassium channels. These are beta-bungarotoxin and crotoxin. And finally, a potent antagonist of one on the serotypes of botulinum neurotoxin (type A) is a broadspectrum potassium channel blocker (4-Aminopyridine and its analogues).

These various findings rightly focus attention on the potassium channel, but it must also be noted that the situation appears to be quite complex. This is due to the non-homogeneity of potassium channels, and it is also due to a series of unexplained and apparently anomalous findings. A consideration of both is essential to the ongoing research.

Work on potassium channels has now revealed that there are at least four major types of ion flow that can be

identified, and to some extent these classes can be subdivided. The four major classes are: i.) resting flux of potassium, ii.) a voltage-dependent, rapid flux that is inactivated, iii.) a voltage-dependent, slower flux that is not inactivated, and iv.) a calcium-dependent flux. In at least one case there is a strong interdependence. The voltage-dependent, rapid flux of potassium leads secondarily to opening of calcium channels. Calcium that reaches the cytosol then triggers the so-called calcium-dependent potassium flux.

An element of complexity enters the picture because the major classes of ion channels can be further subdivided. For example, slow potassium flux is very probably composed of at least two components. As another example, the channels that mediate a particular type of flux in one cell (e.g., voltage-dependent, rapid inactivating flux) may not be identical to the channels that mediate this type of flux in another cell. The common assumption among molecular biologists studying these channels is that they have all descended from a common ancestral gene, but there has been significant divergence with time. Also, the characteristics of individual potassium channels may be modified or even governed by the type of membrane in which they reside.

The complexities inherent in potassium channels are equaled by the seemingly anomalous findings that relate these channels to the actions of toxins that block.

exocytosis. This point was stressed during the last report, and the three most glaring anomalies were cited.

- 4-Aminopyridine and its analogues, by virtue of being potassium channel blockers, can antagonize botulinum neurotoxin type A, but they have much lesser or no ability to antagonize the other serotypes of botulinum toxin or tetanus toxin.

- Dendrotoxin, purportedly by virtue of being a potassium channel blocker, protects tissues against certain phospholipase A2 neurotoxins (e.g., beta-bungarotoxin), but it does not protect against other PLA2 neurotoxins (e.g., crotoxin).

- The data also reveal a crossed anomaly. 4-aminopyridine and its analogues can protect against one serotype of botulinum toxin, but it has not been shown to protect against any of the PLA2 neurotoxins; conversely, dendrotoxin protects against beta-bungarotoxin, but it affords no protection against any of the clostridial neurotoxins (last report).

It must now be reported that there is another unusual quality to the data on interactions. As just discussed, 4-aminopyridine and its analogues do not protect against PLA2 neurotoxins. However, they can potentiate the action of these neurotoxins. As described below, the result is absolutely dependent on the rate of nerve stimulation.

The results obtained by the Principal Investigator and by others show that potassium channels are central to the

study of presynaptic toxins. Unfortunately, it is unclear how channel function relates to toxin action. This is in part due to the absence of a methodology that is designed to characterize channels and to unravel the anomalies that have been discussed. Therefore, the past reporting period has been devoted to an effort to learn and master a new technique for studying potassium channels in situ.

B. Methods

Over a number of years Blaustein and his colleagues at the University of Maryland have developed techniques for studying the flow of ions across the membranes of isolated nerve endings. Their methods have involved the study of ions of interest (i.e., calcium), substitute ions that mimic those ordinarily associated with the action potential or with exocytosis (i.e., rubidium), and the monitoring of dyes that are indicators of cytoplasmic ion concentration (Blaustein and Goldring, 1975; Nachshen and Blaustein, 1982; Blartschat and Blaustein, 1985). During the past Quarter investigators in this contract have collaborated with those in another to build an apparatus that would allow them to mimic the techniques used by Blaustein and his associates.

The procedure was to proceed through three steps. Initially a commercially available, small scale apparatus was purchased and modified. This apparatus was used in preliminary experiments to determine whether ion flux could be measured that was comparable to that previously reported.

Next, a protein toxin was tested on the small scale apparatus, again to ensure that previously reported results could be obtained. Dendrotoxin was used as the test poison, as described by Benishin et al. (1988). Finally, an apparatus for large scale studies was designed and built at Jefferson.

The majority of the reporting period was devoted to building and testing the apparatus for studying ion flux. However, two other projects simultaneously went forward: i.) the study of toxins and channel blockers on phrenic nerve-hemidiaphragm preparations, and ii.) the establishment of a joint, international project (Madison, WI; London, GB; and Philadelphia, PA) to resolve a disputed point in the literature (see below).

C. Results

1. Rubidium Flux Experiments

The initial work with the commercially available apparatus and with dendrotoxin went well. Therefore, the results summarized here will deal with the apparatus that was built at Jefferson and with the data obtained using it.

The apparatus possesses 24 wells (3x8), each of which is capable of holding working volumes of 10 μ l to 1500 μ l. The apparatus can be used with any whole cell or re-sealed cell (e.g., synaptosome) preparation.

The essence of the procedure is that cells are preloaded with the isotope or dye of interest. In the

present case, ^{86}Rb has been used as a marker for potassium flux. After being loaded, the cells are placed in the chambers of the apparatus and washed by filtration to remove unbound ion. The cells are retained by filters at the base of the top plate; the effluent can be directed either into collection vials or into a dump tube.

Typical experiments are conducted over an interval of 60 seconds. In the absence of calcium or depolarizing amounts of potassium, one can monitor resting efflux. The existence of calcium-dependent potassium flux is measured by the difference in efflux in depolarizing medium with calcium and the same medium without calcium. The distinction between the rapid, inactivating flux and the slow, non-inactivating flux is determined graphically by measuring ion flux over time; rapid flux inactivates within less than 10 seconds, but the slow flux continues throughout the experiment.

Our results have shown that all four times of flux can be monitored with ^{86}Rb (rat brain synaptosomes). In quantitative terms, the relative amounts of flux for the four components were:

Resting	-17%
Calcium-dependent	-20%
Rapid, inactivating	-23%
Slow, non-inactivating	-40%

Dendrotoxin was examined for its effects on flux in synaptosomal preparations. Within the concentration range

of 10 to 1000 nM, it acted preferentially on the rapid, inactivation flux. This is in keeping with previous work done by electrophysiologists.

2. Aminopyridines and PLA2 neurotoxins

In the previous report, data were provided that show that 4-AP does not antagonize the onset of neuromuscular blockade caused by beta-bungarotoxin. This finding would appear to be at odds with data reported by Chang and Su (1980). These authors did not find protection, but they did report a notable potentiation. When tested in the range of 10^{-5} to 10^{-4} M, 4-aminopyridine enhanced the rate of onset of beta-bungarotoxin-induced neuromuscular blockade.

Chang and Su (1980) and the present investigator have used similar concentrations of aminopyridine. However, the two laboratories have employed at least three differing techniques: physiological salt solution, rate of nerve stimulation, and concentration of beta-bungarotoxin. The salt solution was thought least likely to contribute to the dissimilar results. Therefore, toxin concentration and rate of nerve stimulation were varied. The results (Table 1) show that toxin concentration was a minor contributor; the rate of nerve stimulation was the major factor.

It would appear that Chang and Su (1980) and the author are both correct. Aminopyridines do not protect against beta-bungarotoxin, but they can produce

potentiation. The latter is a nerve activity-dependent phenomenon.

Table 4

Latin-square evaluation of toxin concentration
and rate of nerve stimulation

	<u>0.1 Hz</u>	<u>1.0 Hz</u>
Beta-bungarotoxin (1×10^{-7} M)	220±19	191±16
Beta-bungarotoxin (1×10^{-6} M)	122±9	108±6
Beta-bungarotoxin (1×10^{-7} M) + 4-AP (50 μ M)	174±16	185±14
Beta-bungarotoxin (1×10^{-6} M) + 4-AP (50 μ M)	95±5	101±7

The data represent the mean±SEM of five preparations.
The values are expressed in minutes.

References

- Bartschat, D.K. and Blaustein, M.P. (1985). Potassium channels in isolated presynaptic nerve terminals from rat brain. *Journal of Physiology* 361, 419-440.
- Benishin, Christina, Sorensen, Roger, Brown, William, Krueger, Bruce and Blaustein, Mordecai (1988). Four Polypeptide Components of Green Mamba Venom Selectively block Certain Potassium Channels in Rat Brain Synaptosomes. *Molecular Pharmacology* 34, 152-159.
- Blaustein, M.P. and Goldring, J.M. (1975). Membrane potentials in pinched-off presynaptic nerve terminals monitored with a fluorescent probe: evidence that synaptosomes have potassium diffusion potentials. *Journal of Physiology* 247, 589-615.
- Chang, C.C. and Su, M.J. (1980). Effect of 3,4-Diaminopyridine and Tetraethylammonium on the Presynaptic blockade caused by B-Bungarotoxin. *Toxicon* 18, 481-484.
- Nachshen, D.A. and Blaustein, M.P. (1982). The influx of calcium, strontium and barium in presynaptic nerve endings. *Journal of General Physiology* 79, 1065-1087.

DISTRIBUTION LIST

5 copies

Commander
US Army Medical Research Institute of
Infectious Diseases
ATTN: SGRD-UIZ-M
Fort Detrick, Frederick, MD 21701-5011

1 copy

Commander
US Army Medical Research and Development
Command
ATTN: SGRD-RMI-S
Fort Detrick, Frederick, Maryland
21701-5012

2 copies

Defense Technical Information Center
(DTIC)
ATTN: DTIC-FDAC
Cameron Station
Alexandria, VA 22301-6145

1 copy

Dean
School of Medicine
Uniformed Services University of the
Health Sciences
4301 Jones Bridge Road
Bethesda, MD 20814-4799

1 copy

Commandant
Academy of Health Sciences, US Army
ATTN: AHS-CDM
Fort Sam Houston, TX 78234-6100